Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 5739–5743, August 1990
Cell Biology

Rescue of a paralyzed-flagella mutant of *Chlamydomonas* by transformation

(radial spokes/flagellar genes/motility)

DENNIS R. DIENER*, ALICE M. CURRY*, KARL A. JOHNSON*, BENJAMIN D. WILLIAMS*†, PAUL A. LEFEBVRE‡, KAREN L. KINDLE§, AND JOEL L. ROSENBAUM*¶

*Department of Biology, Yale University, New Haven, CT 06511; ‡Department of Genetics and Cell Biology, University of Minnesota, Saint Paul, MN 55108; and §Plant Science Center, Biotechnology Building, Cornell University, Ithaca, NY 14853

Communicated by Vincent T. Marchesi, May 7, 1990

ABSTRACT **The biflagellate alga *Chlamydomonas* has been used extensively in the genetic and biochemical analysis of flagellar assembly and motility. We have restored motility to a paralyzed-flagella mutant of *Chlamydomonas* by transforming with the corresponding wild-type gene. A nitrate reductase-deficient paralyzed-flagella strain, *nit1-305 pf-14*, carrying mutations in the genes for nitrate reductase and radial spoke protein 3, was transformed with wild-type copies of both genes. Two-thirds of the cells that survived nitrate selection also regained motility, indicating that they had been transformed with both the nitrate reductase and radial spoke protein 3 genes. Transformants typically contained multiple copies of both genes, genetically linked to each other, but not linked to the original mutant loci. Complementation of paralyzed-flagella mutants by transformation is a powerful tool for investigating flagellar assembly and function.**

The biflagellate alga *Chlamydomonas* is one of the principal eukaryotes used for genetic analysis of flagellar biogenesis and motility. Many mutants with defects in flagellar assembly have been isolated, including cells with flagella of aberrant number (1–3), size (4, 5), and shape (5), as well as mutants with more subtle assembly defects. These include flagella with a reduced beat frequency (6, 7) or abnormal waveform (6, 7) or flagella that are completely paralyzed (8) due to faulty assembly of macromolecular components of the axoneme, such as the dynein arms (6, 9) or the radial spokes (8). The individual polypeptides defective in some of these mutants have been identified (8) and their genes have been cloned (10). Therefore, it should now be possible to complement these mutations by transforming *Chlamydomonas* (11–14) with the corresponding wild-type genes. The ability to transform *Chlamydomonas*, coupled to the ease with which diverse flagellar mutants can be isolated, makes this organism ideally suited for the genetic dissection of flagellar assembly and function.

Flagellar motility is generated by the dynein-based sliding of adjacent outer-doublet microtubules against one another (15). Equally important are the radial spokes, which are involved in the regulation of this sliding to give the flagellar beat its characteristic form (16, 17). Much of what we know about the radial spokes comes from the comparative biochemical and genetic analysis of paralyzed mutants (8, 18). In wild-type cells, radial spokes are attached in pairs along the A-subfiber of each outer-doublet microtubule (19) but are completely absent from the paralyzed flagella of the mutant *pf-14* (8, 18). This strain has a nonsense mutation in the gene for radial spoke protein 3 (RSP3) (10), 1 of 17 polypeptides that make up the radial spokes (18), so that no intact RSP3 is synthesized, radial spokes do not assemble, and the flagella are paralyzed. In this report we describe the transformation of a paralyzed-flagella mutant of *Chlamydomonas*, *pf-14*, with the wild-type RSP3 gene to obtain cells with normal flagellar motility.

MATERIALS AND METHODS

Cell Strains and Culture. The radial-spokeless paralyzed-flagella mutant *pf-14* was obtained from the *Chlamydomonas* Genetics Center (Duke University, Durham, NC). Wild-type strain 21gr and the nitrate reductase (NR) mutant *nit1-305*, as well as culture and genetics techniques, were as described (11).

Transformation. The double mutant *nit1-305 pf-14* was transformed using biolistic techniques (11, 20). The *Chlamydomonas* wild-type genes for NR and RSP3 used for transformation were contained on separate plasmids: pMN24 (21), which contains the NR gene on a 14.5-kilobase insert of genomic DNA; and pRSP3-EB, which contains the RSP3 gene on a 6.6-kilobase *Eco*RI–*Bam*HI fragment from EMBL4 genomic clone 8D (10). *nit1-305 pf-14* cells were plated on medium containing nitrate as the sole source of nitrogen (11) and bombarded with tungsten particles coated with pMN24 and pRSP3-EB. After bombardment, cells that grew on nitrate plates were transferred to liquid culture and examined microscopically for motility.

In all experiments pMN24 was used as a supercoiled plasmid, whereas pRSP3-EB was linearized at the 5′ or 3′ end of the gene with *Bam*HI or *Eco*RI or digested with both enzymes. The mass ratio of pMN24 to pRSP3-EB was varied from 0.2 to 1.0 with a total of 0.5–1.0 μg of DNA used per bombardment. Transformants were obtained under every condition used.

Microscopy. Cells were photographed on Tri-X film through darkfield optics. Exposures were for 1 sec with stroboscopic illumination (Strobex system 236, Chadwick-Helmuth, El Monte, CA) with a flash rate of 5 Hz. For electron microscopy, flagellar microtubules were negatively stained and examined as described (22).

Electrophoresis of Flagellar Proteins. Two-dimensional gel electrophoresis was carried out on isolated flagella as described (23).

DNA Isolation and Southern Blot Analysis. DNA was isolated from *Chlamydomonas* by either of two methods. The first method consisted of lysing cells with SDS and purifying the DNA by centrifugation on a cesium chloride gradient (24). The second method used hexadecyltrimethylammonium bromide (Sigma) to precipitate nucleic acids, in a procedure

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: NR, nitrate reductase; RSP3, radial spoke protein 3.

†Present address: Department of Genetics, Washington University School of Medicine, Saint Louis, MO 63110.
¶To whom reprint requests should be addressed.

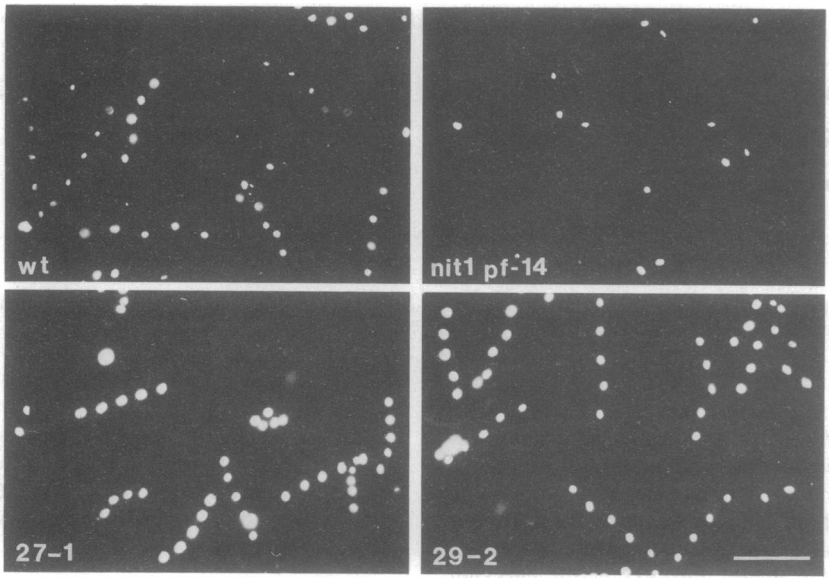

FIG. 1. Light microscopy of transformed cells. Wild-type (wt), *nit1-305 pf-14*, and transformed cells (27-1 and 29-2) were photographed using darkfield optics with exposure times of 1 sec and stroboscopic illumination with a flash rate of 5 Hz. Swimming cells are seen as a set of five bright spots. The stationary *nit1-305 pf-14* cells are seen as single spots. (Bar is 100 μm.)

designed to purify DNA from plant material with a high polysaccharide content (25).

For Southern blot analysis, DNA was digested with *Pvu* II, and approximately 3 μg of each sample was loaded on 0.8% agarose gels. After electrophoresis the DNA was transferred to Nytran sheets (Schleicher & Schuell). Blots were probed for the NR gene with the nick-translated *Kpn* I–*Sal* I fragment from pMN24 or for the RSP3 gene with the *Eco*RI–*Bam*HI fragment from pRSP3-EB. These probes consist of the entire *Chlamydomonas* sequence used in the transformation experiments.

Radioactivity on Southern blots was quantified using a Betascope 603 blot analyzer (Betagen, Waltham, MA). To estimate the number of copies of the RSP3 gene present in transformants, the radioactivity present in an internal fragment was compared between DNA from *nit1-305 pf-14* and transformed cells. To compensate for differences in sample loading, the blot was rehybridized with an α-tubulin probe and the cpm were normalized relative to an α-tubulin band.

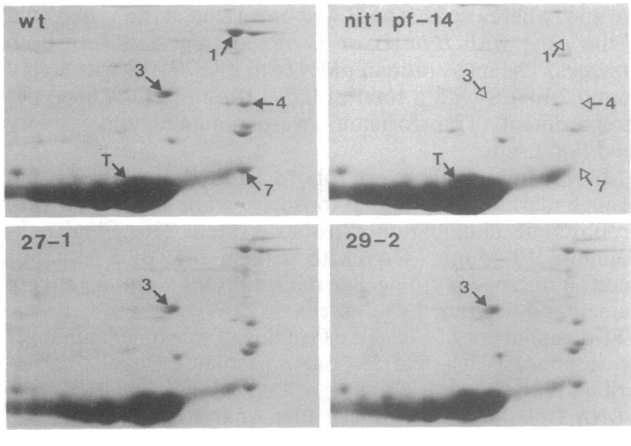

FIG. 2. Electrophoretic analysis of flagella from transformed cells. Flagella from wild-type (wt), *nit1-305 pf-14*, and transformed cells (27-1 and 29-2) were isolated and analyzed by two-dimensional gel electrophoresis (23). The acidic end of the isoelectric focusing dimension is toward the right. The second dimension was SDS/polyacrylamide gel electrophoresis. Only the region of the gel around RSP3 is shown. The solid arrows point to tubulin (T) and radial spoke proteins numbered according to Piperno *et al.* (18). The open arrows show where the same proteins would be, were they present in *nit1-305 pf-14* cells.

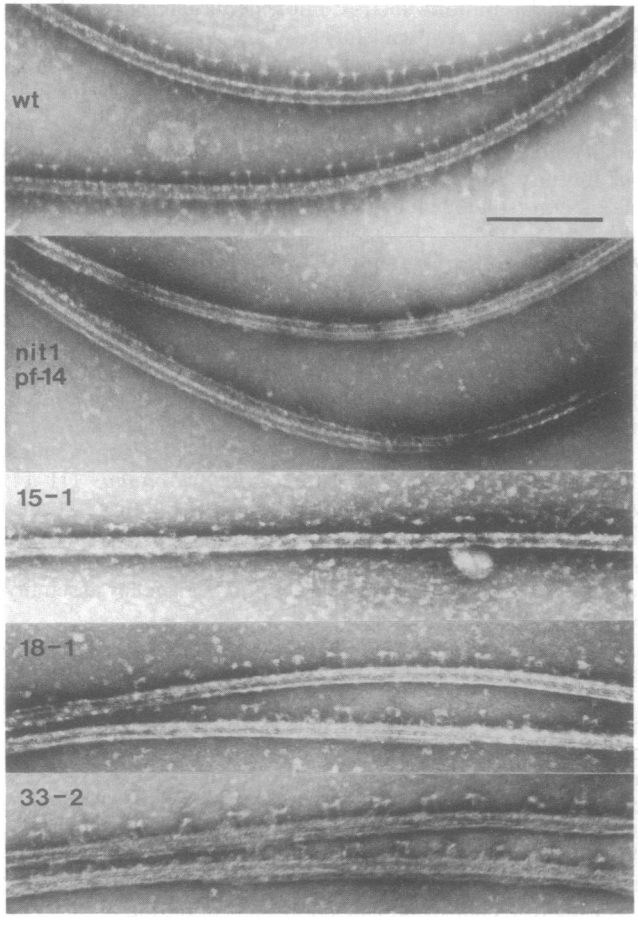

FIG. 3. Electron microscopy of flagella from transformed cells. Outer-doublet microtubules of wild-type (wt), *nit1-305 pf-14*, and transformed cells (15-1, 18-1, and 33-2) were negatively stained and examined by electron microscopy. The paired radial spokes can be seen extending upward from horizontally arranged outer-doublet microtubules. (Bar is 0.2 μm.)

RESULTS

Transformation of *nit1-305 pf-14*. Our goal was to restore motility to the radial-spokeless paralyzed-flagella mutant *pf-14* by transformation with the wild-type RSP3 gene. To facilitate screening for motile transformants a double mutant, *nit1-305 pf-14*, was constructed that also carried a mutation in the gene for NR (26). Because of this mutation these cells cannot survive on medium containing nitrate as the sole source of nitrogen. The wild-type *Chlamydomonas* genes for NR and RSP3, contained on separate plasmids, were introduced into the double mutant by using microprojectile bombardment (11, 20). Cells that had been transformed with the NR gene were selected by their ability to grow on nitrate plates, and these were then examined microscopically to identify motile cells that had been cotransformed with the RSP3 gene. From a total of 24 bombardments (5×10^7 cells per bombardment), 21 colonies were obtained that survived on selective medium; of these, 14 had also recovered motility. As a control, cells were bombarded with particles coated with only the NR gene; none of the 7 nit^+ colonies that were screened contained motile cells.

Phenotype of Transformants. Observation of the motile transformants with light microscopy showed that they swam normally (Fig. 1). Two-dimensional gel electrophoretic analysis of the flagellar proteins from transformed cells indicated that RSP3 and other radial spoke proteins, absent from the flagella of mutant cells, were present in the flagella of transformants (Fig. 2) and that the ratio of RSP3 to other flagellar proteins was normal. Electron microscopy of negatively stained microtubules from splayed flagellar axonemes showed that radial spokes, completely absent from the flagella of mutant cells (Fig. 3 *nit1 pf-14*), were present in pairs along the outer-doublet microtubules of motile transformants (Fig. 3 *15-1*, *18-1*, and *33-2*), as is characteristic of wild-type flagella (Fig. 3 *wt*) (19).

Presence of Introduced DNA in Transformants. DNA from all of the transformants was analyzed on Southern blots; 10 representative examples are shown in Fig. 4. A *Pvu* II digest of DNA from *nit1-305 pf-14* cells produces three prominent fragments that hybridize to the NR gene (21): two fragments contained completely within the gene (Fig. 4 *A* and *C*, bands A and C) and one from the 5′ end of the gene (Fig. 4 *A* and *C*, band B). The 3′ end of the gene produces a small fragment not detected on these blots. In addition to these 3 bands, DNA from transformants typically had one to three extra fragments that hybridized to the NR probe. Several transformants (one example, 27-1, is shown in Fig. 4*C*) had as many as 15 extra bands, suggesting that many copies of the NR gene were present in these cells.

In a few cases, no extra fragments that hybridized to the NR probe were detected (e.g., 29-2 and 18-1), raising the

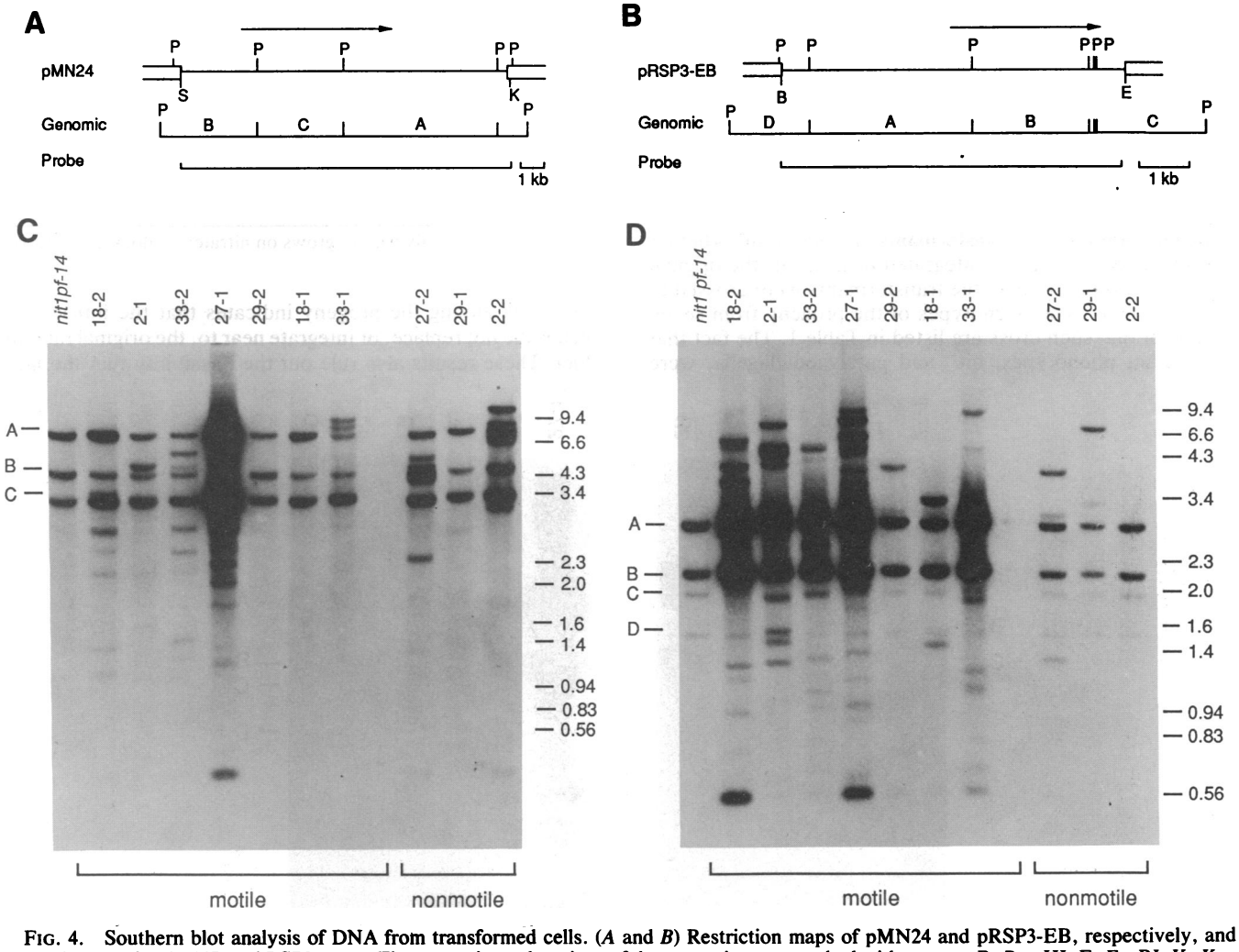

FIG. 4. Southern blot analysis of DNA from transformed cells. (*A* and *B*) Restriction maps of pMN24 and pRSP3-EB, respectively, and genomic maps for the NR and RSP3 genes. The approximate locations of the transcripts are marked with arrows. B, *Bam*HI; E, *Eco*RI; K, *Kpn* I; P, *Pvu* II; S, *Sal* I. (*C* and *D*) Southern blot analysis of the DNA from *nit1-305 pf-14* and transformed cells. DNA was digested with *Pvu* II, and approximately 3 μg of each sample was loaded on 0.8% agarose gels. Blots were probed for the NR gene with the nick-translated *Kpn* I–*Sal* I fragment from pMN24 (*C*) or for the RSP3 gene with the *Eco*RI–*Bam*HI fragment from pRSP3-EB (*D*). The letters marking bands correspond to the fragments shown on the restriction maps in *A* and *B*. The size markers are listed in kilobase pairs.

possibility that these cells were revertants rather than true transformants. This possibility seems unlikely, however, for several reasons. (*i*) Both clones contain extra fragments that hybridize to the RSP3 gene (Fig. 4*D*), indicating that exogenous DNA entered the cells. It is very unlikely that these cells are *nit 1* revertants that were transformed with only the RSP3 gene, since both events occur at a very low frequency. (*ii*) Genetic evidence (see below) rules out the possibility of intragenic reversion for 29-2. (*iii*) In these cells, as with all transformants, the increased intensity of the internal bands (bands A and/or C in Fig. 4*C*) relative to the flanking band (band B) indicates that extra copies of the coding region of the NR gene are present. It is possible that, in these transformants, nonessential DNA flanking the NR gene was lost, so that, as for the 3′ end of the endogenous gene, the flanking *Pvu* II fragments were too small to be detected on these gels.

When DNA from the same transformants was probed with the RSP3 gene, all the cells that had regained motility also contained new copies of the RSP3 gene as shown by the increased intensity of the internal bands A and B (Fig. 4*D*) and by the presence of extra bands hybridizing to the RSP3 gene probe. In the seven motile transformants shown in Fig. 4, the number of introduced copies of the RSP3 gene incorporated into transformants varied from 1 to 20. Even some of the nit$^+$ transformants that remained paralyzed contained extra fragments that hybridized to the RSP3 gene (27-2 and 29-1, Fig. 4*D*). In these cases possibly only a fragment of the RSP3 gene had integrated or the gene had integrated into a location where it could not be efficiently expressed.

When these same blots were probed with sequences that recognized only the vector sequences in pMN24 and pRSP3-EB, many of the same bands that hybridized to probes for the introduced genes also hybridized to the vector. This indicates that at least part of the vector was retained by the cells along with the introduced *Chlamydomonas* sequences (data not shown).

Genetic Analysis of Transformants. To determine whether the introduced DNA had integrated near, or at, the original *nit 1* or *pf-14* loci, five motile transformants were crossed to wild-type cells. The phenotypes of the progeny from seven tetrads of one such cross are listed in Table 1. The fact that the mutant phenotypes, nit$^-$ and paralyzed flagella, were observed among the progeny indicates that the introduced genes did not replace, or integrate near to, the original mutant loci. These results also rule out the possibility that the nit$^+$

Table 1. Phenotypes of the products of seven complete tetrads obtained in a cross of transformant 27-1 with wild-type cells

Tetrad progeny	Growth on nitrate	Motility
1A	+	S
1B	+	P
1C	+	S
1D	−	P
2A	+	S
2B	+	P
2C	+	S
2D	+	S
3A	−	S
3B	+	S
3C	+	S
3D	−	S
4A	+	S
4B	+	S
4C	+	S
4D	+	S
5A	−	S
5B	+	S
5C	−	S
5D	+	S
6A	+	S
6B	+	S
6C	+	S
6D	−	S
7A	+	P
7B	+	S
7C	+	P
7D	+	S

S, swims; P, paralyzed; +, grows on nitrate; −, does not grow on nitrate.

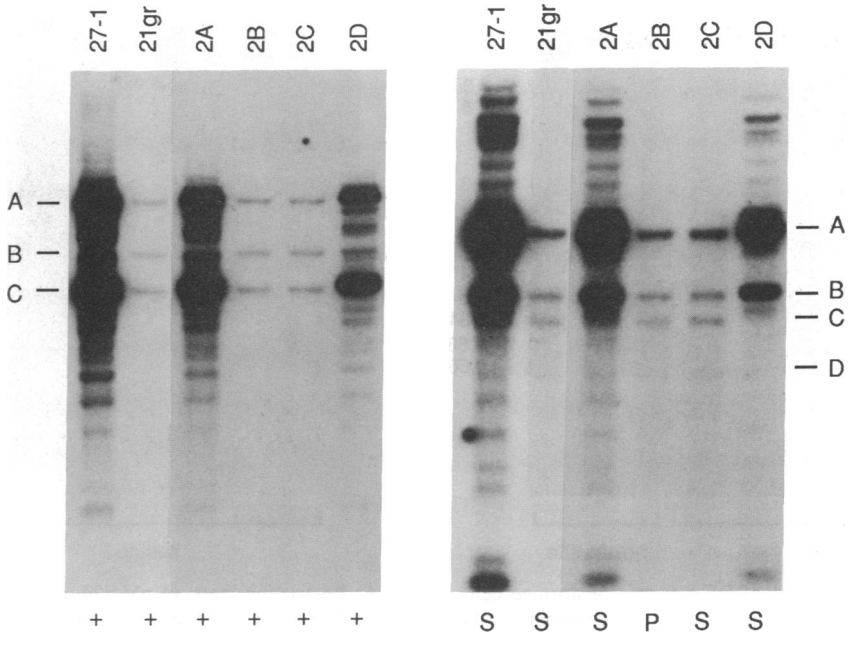

FIG. 5. Southern blot analysis of DNA from a tetrad from a cross of wild-type cells with transformant 27-1. Southern blots were probed for the genes for NR (*Left*) or RSP3 (*Right*). Methods were the same as for Fig. 4. +, Grows on nitrate as sole source of nitrogen; −, does not grow on nitrate; S, swims; P, paralyzed.

and swimming phenotypes resulted from intragenic reversion of the *nit1-305* or *pf-14* mutations. Both mutant phenotypes were recovered from each of four crosses of transformants to wild-type cells. In the fifth case (33-2), however, 79 out of 80 progeny were nit$^+$. This suggests that in this case either the introduced genes are linked to the *nit1* locus or several copies of the NR gene integrated into different chromosomes and do not segregate together.

The results of these crosses also suggest that the functional copies of the two introduced genes are linked to each other. When the products of a tetrad include two motile and two nonmotile cells (e.g., tetrad 1A–1D; Table 1), both motile cells must contain the RSP3 gene from the wild-type parent and the introduced RSP3 genes. If the introduced NR gene is linked to the introduced RSP3 gene, these two motile cells must also be nit$^+$. Likewise, if only two cells of a tetrad are nit$^+$, they will also be motile if the introduced genes are linked to each other. Twenty-six complete tetrads were identified in the crosses described above in which only two of the progeny were motile or only two were nit$^+$. In all 26 cases, the two cells that were wild type for one trait were also wild type for the other, strongly suggesting that all the introduced genes were tightly linked.

Further evidence for linkage of the introduced genes was obtained by following the segregation of the introduced genes among the tetrad progeny. The DNA from three tetrads from each of two crosses was analyzed on Southern blots (one example is shown in Fig. 5). In each of the tetrads examined, only two types of cells were found: cells that contained only the parental copies of both genes, and cells that contained all the extra copies of both introduced genes. The fact that all the extra fragments of both introduced genes segregate together provides additional evidence that these genes are linked.

DISCUSSION

The ability to use transformation to complement flagellar mutations with wild-type genes will be useful in the investigation of the molecular basis of flagellar assembly and function. *Chlamydomonas* strains have been identified with mutations in the genes for several of the radial spoke proteins (8) and tubulins (ref. 27, and C. D. Silflow, S. W. James, and P.A.L., unpublished observation). Many other mutants have been isolated that are defective in the assembly of inner (8) or outer (6, 9) dynein arms; some of these strains are likely to have mutations in dynein genes. With the cloning of *Chlamydomonas* genes for radial spoke proteins (10, 31), tubulins (28, 29), and dyneins (30, 31), many of these mutants can be transformed with the corresponding wild-type genes. By modifying these genes to alter specific regions of the encoded polypeptide, the importance of specific polypeptide domains in the targeting, assembly, and function of these proteins in flagellar biogenesis and motility can be determined. Similar techniques can be used to study elements that regulate gene expression during flagellar regeneration. In addition, there are many flagellar mutants for which specific gene products have not yet been identified. With the development of a simple and more efficient method for transformation of *Chlamydomonas* by agitating cells in the presence of DNA and glass beads (14), it should be possible to isolate the genes responsible for these traits can by complementation.

We thank Inna Golub for excellent technical assistance. This work was supported by National Institutes of Health Grants GM 14642 (J.L.R.) and GM 34437 (P.A.L.), by U.S. Department of Agriculture Competitive Research Grant 37234-4007 (P.A.L.), and by the Cornell National Science Foundation Plant Science Center, a unit in the U.S. Department of Agriculture-U.S. Department of Energy-National Science Foundation Plant Science Centers Program and a unit of the Cornell Biotechnology Program, which is sponsored by the New York State Science and Technology Foundation, a consortium of industries, and the U.S. Army Research Office.

1. Huang, B., Ramanis, Z., Dutcher, S. K. & Luck, D. J. L. (1982) *Cell* **29**, 745–753.
2. Goodenough, U. W. & St. Clair, H. S. (1975) *J. Cell Biol.* **66**, 480–491.
3. Kuchka, M. R. & Jarvik, J. W. (1982) *J. Cell Biol.* **92**, 170–175.
4. McVittie, A. (1972) *J. Gen. Microbiol.* **71**, 525–540.
5. Jarvik, J. W. & Chojnacki, B. (1985) *J. Protozool.* **32**, 649–656.
6. Mitchell, D. R. & Rosenbaum, J. L. (1985) *J. Cell Biol.* **100**, 1228–1234.
7. Brokaw, C. J. & Kamiya, R. (1987) *Cell Motil. Cytoskel.* **8**, 68–75.
8. Huang, B. P.-H. (1986) *Intl. Rev. Cytol.* **99**, 181–215.
9. Kamiya, R. (1988) *J. Cell Biol.* **107**, 2253–2258.
10. Williams, B. D., Velleca, M. A., Curry, A. M. & Rosenbaum, J. L. (1989) *J. Cell Biol.* **109**, 235–245.
11. Kindle, K. L., Schnell, R. A., Fernández, E. & Lefebvre, P. A. (1989) *J. Cell Biol.* **109**, 2589–2601.
12. Debuchy, R., Purton, S. & Rochaix, J.-D. (1989) *EMBO J.* **8**, 2803–2809.
13. Mayfield, S. P. & Kindle, K. L. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 2087–2091.
14. Kindle, K. L. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 1228–1232.
15. Gibbons, I. R. (1981) *J. Cell Biol.* **91**, 107–124.
16. Witman, G. B., Plummer, J. & Sander, G. (1978) *J. Cell Biol.* **76**, 729–747.
17. Brokaw, C. J., Luck, D. J. L. & Huang, B. (1982) *J. Cell Biol.* **92**, 722–733.
18. Piperno, G., Huang, B., Ramanis, Z. & Luck, D. J. L. (1981) *J. Cell Biol.* **88**, 73–79.
19. Hopkins, J. M. (1970) *J. Cell Sci.* **7**, 238–239.
20. Klein, T. M., Wolf, E. D., Wu, R. & Sanford, J. C. (1987) *Nature (London)* **327**, 70–73.
21. Fernández, E., Schnell, R., Ranum, L. P. W., Hussey, S. C., Silflow, C. D. & Lefebvre, P. A. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 6449–6453.
22. Dentler, W. L. & Rosenbaum, J. L. (1977) *J. Cell Biol.* **74**, 747–759.
23. L'Hernault, S. W. & Rosenbaum, J. L. (1983) *J. Cell Biol.* **97**, 258–263.
24. Weeks, D. P., Beerman, N. & Griffith, O. M. (1986) *Anal. Biochem.* **152**, 376–385.
25. Johnson, K. A. & Sussex, I. (1990) *Chromosoma* **99**, in press.
26. Fernández, E. & Cárdenas, J. (1983) *Z. Naturforsch.* **38c**, 439–445.
27. Bolduc, C., Lee, V. D. & Huang, B. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 131–135.
28. Youngblom, J., Schloss, J. A. & Silflow, C. D. (1984) *Mol. Cell. Biol.* **4**, 2686–2696.
29. Silflow, C. D., Chisholm, R. L., Conner, T. W. & Ranum, L. P. W. (1985) *Mol. Cell. Biol.* **5**, 2389–2398.
30. Mitchell, D. R. (1989) *Cell Motil. Cytoskel.* **14**, 435–445.
31. Williams, B. D., Mitchell, D. R. & Rosenbaum, J. L. (1986) *J. Cell Biol.* **103**, 1–11.